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Okadaic acid induces both augmentation and inhibition of opsonized zymosan-stimulated superoxide production by differentiated HL-60 cells. Possible involvement of dephosphorylation of a cytosolic 21K protein in respiratory burst

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Abstract

We found that okadaic acid (OA), a potent tumor promotor and a phosphatase inhibitor, has a unique opposing effect on opsonized zymosan (Op.-zym.)-elicited O_2^- production by differentiated HL-60 cells in a narrow range of concentrations but does not induce any O_2^- production by itself. Okadaic acid magnified the O_2^- production 2.5-fold at 1.0 μM, while it inhibited it at 2.0 μM or higher concentrations. This effect of OA did not correspond to the changes in the expression of surface receptors (CD11b/CD18, CR3) for Op.-zym., because they were weakly down-regulated by OA at any concentration. Two-dimensional gel electrophoresis revealed that in the absence of OA, Op.-zym. induced rapid dephosphorylation of a cytosolic 21K protein with a very slight increase in phosphorylation of membranous $p47^{phox}$, which is one of the cytosolic factors required for respiratory burst. In the presence of a stimulatory concentration (1.0 μM) of OA, the Op.-zym.-caused dephosphorylation of the 21K protein was still observed and the phosphorylation of $p47^{phox}$ was enhanced. In the presence of an inhibitory concentration (2.0 or 5.0 μM) of OA, the Op.-zym.-induced dephosphorylation of the 21K protein was strongly inhibited while $p47^{phox}$ was heavily phosphorylated. Acid hydrolysis of the 21K phosphoprotein yielded only phosphoserine as a phosphoamino acid. Furthermore, at least part of the 21K protein seemed to be associated with $p67^{phox}$ and $p47^{phox}$, because it was co-immunoprecipitated with those cytosolic factors. These results suggest that a cytosolic 21K protein plays an important role in respiratory burst through dephosphorylation by a phosphoserine phosphatase, and that the dephosphorylated 21K protein may work synergistically with the phosphorylated $p47^{phox}$ on the pathway for activation of the respiratory burst oxidase.

Keywords: Superoxide; Okadaic acid; Dephosphorylation; Opsonized zymosan; HL-60 cell

1. Introduction

Superoxide (O_2^-) production is the most essential function of microbicidal leukocytes. The enzyme responsible for the production of the active oxygen is a membrane-bound oxidase that catalyzes the one-electron reduction of molecular oxygen at the expense of NADPH and its mechanisms of activation have been widely investigated [1,2]. Recently, various cytosolic factors have been found to be required for activating the membrane-bound oxidase in vitro and some of them have been sequenced and well characterized [3–8]. In most cases, however, the activities of those factors were tested by an artificial cell-free system, and the physiological mechanisms of the O_2^- production in whole cells are still unclear [1].

In general, protein phosphorylation is one of the most important events in signal transduction through changes in protein–protein interactions and dynamic movement or translocation of functional proteins [9–12]. In the case of

Abbreviations: OA, okadaic acid; Op.-zym., opsonized-zymosan; DMSO, dimethyl sulfoxide; HBSS, Hanks' balanced salt solution; FITC, fluorescein isothiocyanate; PVDF, polyvinylidenedifluoride; SDS, sodium dodecyl sulfate; PAGE; polyacrylamide gel electrophoresis.

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neutrophils, there are also many reports which show importance of protein phosphorylation in $O_2^{\cdot-}$ generation by intact cells (reviewed in Ref. [13]), while the phosphorylation/dephosphorylation system could not be reconstituted in the cell-free system [1,13,14]. $p47^{phox}$, a cytosolic factor, is thought to be translocated from the cytosol to the membrane by phosphorylation [15], whereas the real physiological significance of phosphorylation has not been established. Dephosphorylation of a 20K phosphoprotein was first reported by Andrews and Babior [16] in response to a number of particulate and non-particulate stimuli including opsonized-zymosan (Op.-zym), PMA, formyl-Met-Leu-Phe, digitonin, and NaF. Though dephosphorylation of low molecular weight protein(s) was later confirmed by others [17–19], most reports have focused on the increased phosphorylation of known NADPH-oxidase components. However, dephosphorylation of phosphoproteins should be controlled strictly to maintain homeostasis in the $O_2^{\cdot-}$-producing cells. On the other hand, okadaic acid (OA), a potent tumor promotor [20] and a phosphatase inhibitor [21], was found to have an enhancing effect on activation of neutrophils stimulated with arachidonic acid [22], phorbol ester combined with an inhibitor for protein kinase C [23] and formyl-Met-Leu-Phe [24–26] and to have an inhibitory effect on phorbol ester activation [24,25].

In this paper, we describe a unique dual effect of OA on Op.-zym.-triggered $O_2^{\cdot-}$ production and show evidence suggesting the importance of dephosphorylation of a cytosolic 21K protein for the $O_2^{\cdot-}$ production in intact cells.

2. Materials and methods

2.1. Cells

HL-60 cells obtained from the Japanese Cancer Research Resources Bank were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. The original cells, which were free of mycoplasmas, were expanded and stored in a liquid nitrogen stocker. To maintain the quality of the cells, the frozen cells were thawed every three months and used for experiments. The doubling time of the cells was about 40 h. Log-phase growing cells were harvested and induced to neutrophil-like cells with 1.25% dimethyl sulfoxide (DMSO) for six days according to Collins et al. [27]. When the DMSO-induced cells were stimulated with Op.-zym., their $O_2^{\cdot-}$-producing ability was about 0.7–1.1 nmol/10^6 cells. Cell viability was assayed by the method of dye exclusion using Trypan blue.

2.2. Reagents

Okadaic acid (Wako Pure Chemical Industries, Osaka, Japan) was dissolved to give a concentration of 1.0 mM in DMSO and diluted with Hanks' balanced salt solution (HBSS). Rabbit anti-$p47^{phox}$ antibody and anti-$p67^{phox}$ antibody were prepared by immunizing rabbits with synthesized peptide (340–355 of $p47^{phox}$ and 437–450 of $p67^{phox}$)-conjugated bovine serum albumin and purified by affinity chromatography using the corresponding peptide-fixed Sepharose 6B. Monoclonal anti-Mac-1 (CD11b/CD18) antibody and fluorescein isothiocyanate (FITC)-labeled anti-IgG antibody were purchased from Cosmo-Bio (Tokyo, Japan). Rabbit anti-rac antiserum and recombinant rac protein, a positive marker for immunoblotting, were kindly donated by Drs. K. Kaibuchi and Y. Takai (Kobe University, Japan). Rabbit anti-myosin light chain antiserum was a product of Sigma (St. Louis, MO). Protein G-agarose was obtained from Boehringer Mannheim (Germany). ^{32}Pi was obtained from DuPont/NEN Research Products (Boston, MA). Op.-zym. was prepared according to the method of Markert et al. [28]. Briefly, 700 mg of zymosan A (Sigma) was incubated with 70 ml of human serum at 37° C for 30 min and washed with HBSS. The Op.-zym. suspended in HBSS (10 mg/ml) was divided and stored at −80° C until use.

All other chemicals used were commercial preparations of the highest purity.

2.3. Measurement of $O_2^{\cdot-}$ production

The $O_2^{\cdot-}$ Production was determined by reduction of cytochrome *c* according to Markert et al. [28]. Five hundred microliters of HBSS containing 1.5×10^6 cells and OA was incubated at 37° C for 15 min. After addition of cytochrome *c* (final concentration 1 mg/ml), the cells were stimulated with Op.-zym. (final concentration 1 mg/ml) at 37° C. Time-scan data were obtained by using a spectrophotometer (Hitachi, Model 3200, Tokyo, Japan) equipped with a photomultiplier positioned close to the cuvette, a windmill cell mixer developed by Kakinuma et al. [29] and a temperature controller.

2.4. Changes in surface receptors for Op.-zym

After the cells ($1.5 \cdot 10^6$) were incubated with OA as above, 4 ml of ice-cold PBS was added and the cells were centrifuged ($100 \times g$, 15 min). The pelleted cells were suspended in 50 μl of PBS, 5 μl of monoclonal anti Mac-1 (CD11b) antibody was added, and the mixture was incubated at 25° C for 30 min with gentle shaking. The cells were then washed with 5 ml of ice-cold PBS and resuspended in 50 μl of PBS. To the suspension, 5 μl of FITC-labeled anti-mouse IgG antibody solution was added and the mixture was incubated at 25° C for 30 min in the dark with occasional mixing. After being washed with PBS, the cells were fixed with neutralized paraformaldehyde (final concentration 1.9%) at 25° C for 1 h. The fixed cells were washed with PBS and subjected to flow cytometry (PROFILE, Coulter Electronics, Hialeah, FL).

2.5. Protein phosphorylation of HL-60 cells

The differentiated HL-60 cells, which had been loaded with ^{32}Pi, were preincubated with OA as above and stimulated with Op.-zym. at 37° C, usually for 2 min. After separation of the cytosolic and membranous fractions, two-dimensional gel electrophoresis was performed. The details of the experimental conditions were the same as described before [17]. The ^{32}P incorporated in a protein spot was determined by radioluminography using a Bioimage Analyzer BAS2000 (Fuji Film, Tokyo). To calculate the means of data, each value for 21K protein or $p47^{phox}$ in the absence of OA was normalized. All data were obtained within linearity.

2.6. Electroblotting and analysis of phosphoamino acids

After two-dimensional electrophoresis was performed as described above, the gels (1 mm thick) were rinsed with blotting buffer (10 mM 3-cyclohexylaminopropane sulfonic acid buffer (pH 11.0) containing 10% methanol). The proteins separated on the gels were electrophoretically transferred to polyvinylidenedifluoride (PVDF) membranes (15 × 15 cm, Bio-Rad, Helcules, CA) at 300 mA and 4° C for 2 h. By the electroblotting, more than 95% of 21K protein was recovered on the membranes. The spots of the 21K phosphoprotein, which were identified by autoradiography, were excised from the blots. The 21K protein on the three excised membranes (about 4 mm in diameter), which was derived from about 3×10^7 unstimulated cells, were hydrolyzed in the vapor of concentrated HCl at 110° C for 2 h. Then, the membranes were wetted with 20 μl of methanol, and extracted with 200 μl of distilled water. The extract was dried in an Eppendorf tube by Speed Vac (Savant Instrument, Farmingdale, NY) and solubilized in 20 μl of 3 mM cold phosphoamino acid solution containing orange G (electrophoresis marker). Five microliters of the sample was transferred to a cellulose plate and electrophoresis was performed as described previously [10]. The position of phosphoamino acids were identified by ninhydrin staining.

2.7. Immunoprecipitation

Because the cytosolic fraction prepared as described above contained various inhibitors at high concentrations, which may prevent antibody–antigen interaction, the fraction was diluted four-fold with Tris-buffered saline (TBS). To the diluted fraction (300 μl), antibody-coated protein G-agarose (packed volume 25 μl) was added and mixed at 4° C for 17 h. After being washed five times with 400 μl of TBS using Sample Prep (Japan Millipore), the materials bound to the gel were solubilized with SDS-sample buffer for electrophoresis (50 μl) containing dithiothreitol. The samples were subjected to SDS-PAGE as described previously [12] using 12.5% gel followed by autoradiography.

3. Results

3.1. Effect of OA on $O_2^{\cdot-}$ production by HL-60 cells

Although OA alone did not show any effect on $O_2^{\cdot-}$ production by the HL-60 cells as previously described [22,26], it had a unique biphasic effect on the Op.-zym.-stimulated cells in a narrow range of concentration (Fig. 1). OA strongly enhanced the Op.-zym.-stimulated $O_2^{\cdot-}$ production at 1 μM, whereas it inhibited it at 2 μM or higher concentrations. During the incubation time, OA did not show any significant cytotoxicity, while more than 60 min incubation decreased viability.

Fig. 1 shows the results of a typical end point assay 10 min after stimulation. Then we investigated whether OA affected the rate or duration of the $O_2^{\cdot-}$ production. The time-scanning data (Fig. 2) showed that OA enhanced the rate of $O_2^{\cdot-}$ production at a concentration of 1 μM or less, while it decreased it at 2 μM or higher concentrations. These results indicate that the effect of OA shown in Fig. 1 was derived from the changes in the rate of $O_2^{\cdot-}$ production. This concentration-dependent opposing effect of OA was clearly observed only when Op.-zym., a particulate physiological activator, was used and not when other soluble stimulators including PMA and formyl-Met-Leu-Phe were used (data not shown).

3.2. Effect of OA on receptors for Op.-zym

The receptor known as complement-receptor type 3 (CD11b/CD18) is believed to be the first site of action of Op.-zym. [30]. Since it has been shown that upon stimulation the complement receptors are rapidly translocated

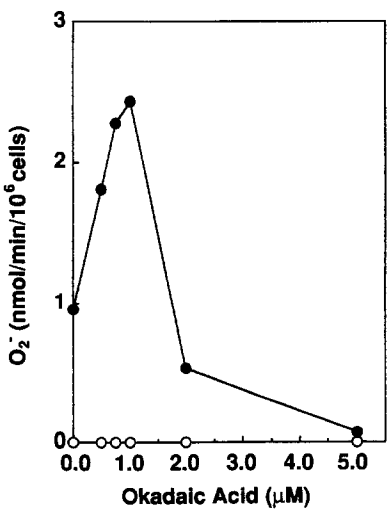

Fig. 1. Effect of okadaic acid on superoxide production by differentiated HL-60 cells. After incubation in the presence of OA at the indicated concentrations for 15 min, the cells were exposed to Op.-zym. (●) or to vehicle (○) for 10 min. The amount of superoxide released was determined by reduction of cytochrome *c*. Experimental details were described in Section 2.

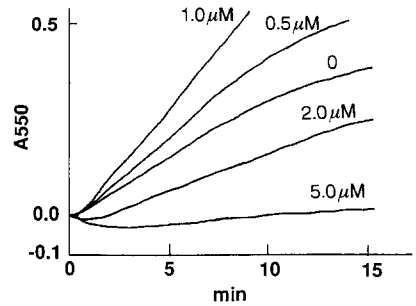

Fig. 2. Time course of superoxide release from okadaic acid-treated HL-60 cells. After DMSO-induced HL-60 cells were incubated with OA as for Fig. 1 at the indicated concentrations, Op.-zym. was added at 0 time and the released $O_2^{\cdot-}$ was monitored by increase in the absorbance at 550 nm of cytochrome *c* at 37° C with mixing.

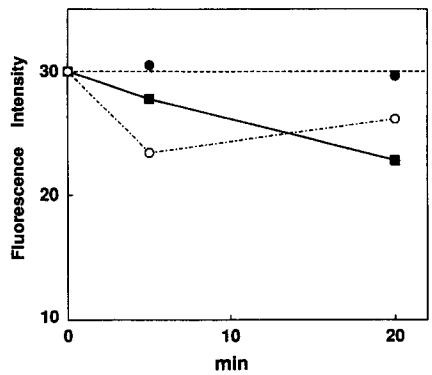

Fig. 3. Effect of okadaic acid on the expression of receptor (CD11b/CD18) for Op.-zym. After incubation with OA at 37° C for the time indicated on the horizontal axis, the cells were treated with monoclonal anti-Mac-1 (CD11b/CD18) antibody followed by FITC-labeled anti-mouse IgG antibody. The fluorescence intensity on the vertical axis, which was measured by a flow cytometer, indicates the relative amount of receptors on the cell surfaces. Experimental details are described in Section 2. ●, HBSS; ■, 1 μM OA; ○, 5 μM OA.

from an intracellular pool to the cell surface [31], and that some tumor promoters cause dynamic changes in various surface receptors [12,32–34], we investigated whether OA induces changes in the expression of the receptors by flow cytometry using monoclonal anti-Mac-1 (CD11b/CD18) antibody. As shown in Fig. 3, a weak down-regulation of the receptors was observed at both concentrations of OA, while the drug did not cause marked changes in the receptors that could explain the biphasic effect shown in Fig. 1.

3.3. Protein phosphorylation

As protein phosphorylation is thought to be important in $O_2^{\cdot-}$ production [13,17] and OA is known to be a potent inhibitor of phosphoprotein phosphatases types 2A and 1 [21], we determined the effect of OA on protein phosphorylation by two-dimensional gel electrophoresis. In the absence of OA, the greatest change in phosphorylation of proteins caused by Op.-zym. was dephosphorylation of the 21K protein, of which isoelectric point (pI) was about 6.5 (Fig. 4, indicated by arrows). The 21K phosphoprotein was observed only in the cytosolic fraction, while the phosphorylated $p47^{phox}$, which is believed to be involved in the $O_2^{\cdot-}$ production triggered by various stimulants [13,17], was observed in both cytosolic and membranous fractions as previously reported [13,15,17]. Under the electrophoresis conditions, $p47^{phox}$ gave at least six spots depending on its phosphorylated states [15,17], while the phosphorylation of $p47^{phox}$ was increased very slightly by Op.-zym. (Fig. 4, indicated between arrowheads). The changes in degree of the phosphorylation was accurately quantified on the 21K protein in the cytosolic fraction and $p47^{phox}$ in the membranous fraction by radioluminography using a Bioimage Analyzer, which was much more sensitive and quantitative than autoradiography for determining the ra-

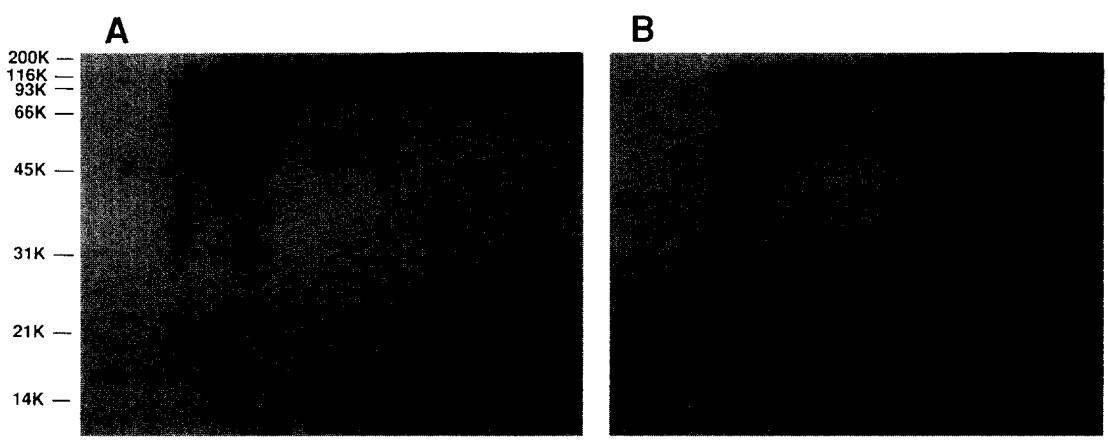

Fig. 4. Dephosphorylation of the 21K protein caused by Op.-zym. ^{32}Pi-loaded HL-60 cells were exposed to HBSS(A) or Op.-zym. (B) 37° C for 2 min. A portion of the cytosolic fraction was subjected to two-dimensional electrophoresis and autoradiography. The arrow in each photograph indicates '21K protein'. The area between the arrowheads is the position of differently phosphorylated $p47^{phox}$, which was identified by the ^{32}P-labeled $p47^{phox}$ of the membranous fraction and was confirmed by immunoblotting using anti-$p47^{phox}$ antibody.

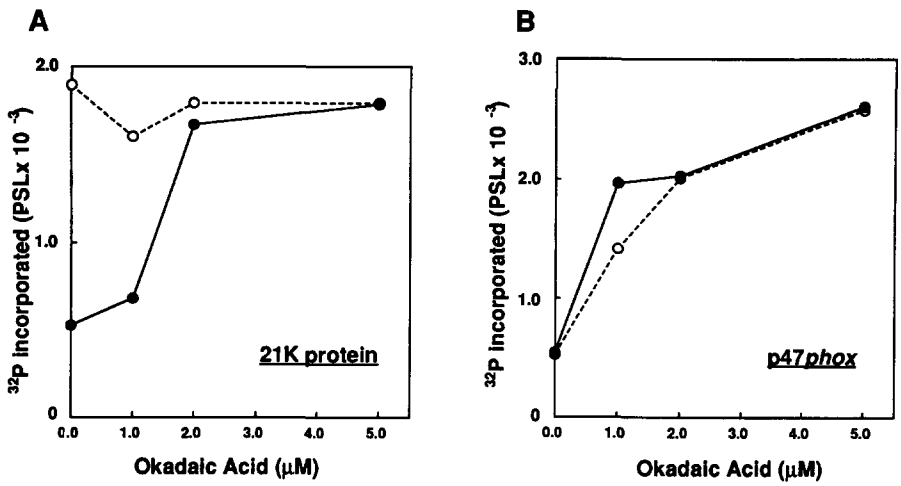

Fig. 5. Effect of okadaic acid on phosphorylation of cytosolic 21K protein and membranous p47^{phox}. ^{32}P incorporated into the 21K protein (A) and p47*phox* (B) on two-dimensional gels was determined by a Bioimage Analyzer. The vertical axes show photon-stimulated luminescence (PSL) which corresponded to the amount of radioactivity of ^{32}P. The horizontal axes show the concentration of OA. ○, not stimulated; ●, stimulated with Op.-zym. Standard deviations are less than 5.8%.

dioisotope (Fig. 5). Phosphorylation of the 21K protein and p47^{phox} was differently affected by OA at stimulatory and inhibitory concentrations. In the presence of a stimulatory concentration (1 μM) of OA, the cytosolic 21K protein was still dephosphorylated while phosphorylation of p47^{phox} was significantly enhanced. In contrast, at inhibitory concentrations (2 μM or higher), OA strongly inhibited the Op.-zym.-caused dephosphorylation of 21K protein. These results suggest that dephosphorylation of the 21K protein is essential for Op.-zym.-triggered $O_2^{\cdot-}$ production and that the OA-caused inhibition of its dephosphorylation resulted in the inhibition of $O_2^{\cdot-}$ production, even if p47^{phox} was heavily phosphorylated. The stimulatory effect of OA (1 μM) on $O_2^{\cdot-}$ production was accompanied by both dephosphorylation of 21K protein and phosphorylation of p47^{phox}. In a hydrolysate of the 21K protein, only phosphoserine was detected (Fig. 6). These results suggest that serine phosphatase, which should be involved in the dephosphorylation of the protein, could be inhibited by OA at 2 μM or higher concentrations.

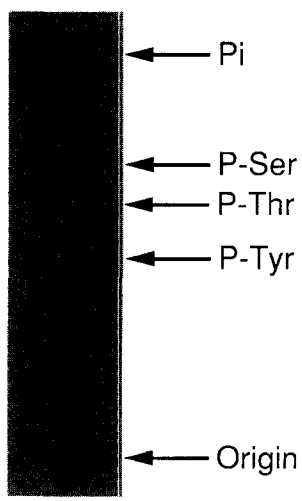

Fig. 6. Analysis of phosphoamino acids of 21K protein. ^{32}P-labeled 21K protein on PVDF membranes was hydrolyzed in the vapor of HCl and extracted with distilled water. The extract was transferred to a cellulose plate and electrophoresis was carried out at pH 3.5. After the positions of phosphoamino acids were identified by ninhydrin staining, autoradiography was performed. Details were given in Section 2. The positions of cold phosphoamino acids and orthophosphate (Pi) are indicated.

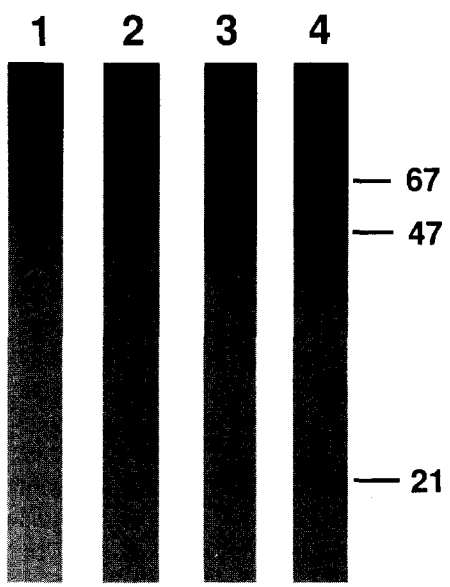

Fig. 7. Coimmunoprecipitation of 21K protein with cytosolic components of respiratory burst oxidase. ^{32}P-labeled proteins absorbed to antibody-coated protein G-agarose were solubilized and analyzed by SDS-PAGE and autoradiography. 1, normal rabbit IgG (control); 2, anti-rac antiserum; 3, anti-p47^{phox} antibody; 4, anti-p67^{phox} antibody. Four similar experiments yielded essentially the same patterns on autoradiograms.

3.4. Immunoprecipitation

To investigate the relationship between the 21K phosphoprotein and the known cytosolic components of $O_2^{\cdot-}$-producing NADPH-oxidase, immunoprecipitation experiments were performed using antibodies against rac protein, $p47^{phox}$, and $p67^{phox}$. Since there were so many phosphorylated proteins in the cytosolic fraction (Fig. 4), nonspecific proteins bound to normal rabbit IgG were observed on the autoradiogram (Fig. 7, lane 1). However, the 21K protein was clearly coprecipitated with the phosphorylated $p67^{phox}$ (lane 4) and significantly with $p47^{phox}$ (lane 3). In contrast, the 21K phosphoprotein was not observed with rac protein, which was required for $O_2^{\cdot-}$ production [7,8] and was not phosphorylated (lane 2). A few other proteins were also observed with $p47^{phox}$ and $p67^{phox}$, but they have not been identified as yet.

Based on the above results, the 21K protein seems to be a phosphoprotein which plays a crucial role in the signal transduction pathway for the Op.-zym.-induced $O_2^{\cdot-}$ production in intact cells.

4. Discussion

We found that OA has an unusual opposing effect on $O_2^{\cdot-}$ production by Op.-zym.-stimulated HL-60 cells in a narrow range of concentrations and studied its mechanisms of action. First, the changes in expression of the receptors for Op.-zym. were investigated. OA caused a weak down-regulation of the receptors at both concentrations tested. The dual effect of OA could not be explained simply by changes in the receptors. Then, protein phosphorylation of Op.-zym.-stimulated cells was studied by two-dimensional gel electrophoresis. Although protein phosphorylation of neutrophils has been well studied by using soluble stimulants [13], there are almost no reports which describe protein phosphorylation of the cells stimulated by insoluble Op.-zym., which is a physiological activator. On the two-dimensional gels, we found a 21K phosphoprotein. Its characteristics were as follows: (1) It is a constitutively phosphorylated protein which was markedly dephosphorylated when the cells were stimulated by Op.-zym. (2) The dephosphorylation was rapid since it was observed within 30 s when the cells were activated by Op.-zym. (3) Phosphoamino acid analysis revealed that the phosphorylated residue was serine. A serine kinase and a serine phosphatase seemed to control the phosphorylated state of the 21K protein. (4) The isoelectric point was estimated to be about 6.5 on two-dimensional gels. (5) The phosphorylated protein seemed to be located in the cytosol, because it was observed only in the cytosolic fraction, not in the membranous fraction. (6) The 21K protein seemed to be different from rac protein and myosin light chain because its position on autoradiograms could not be superposed on their spots on two-dimensional immunoblots (data not shown). (7) According to the results of immunoprecipitation, at least part of the phosphorylated 21K protein seemed to be associated with $p67^{phox}$ and $p47^{phox}$, the cytosolic components required for $O_2^{\cdot-}$ production. (8) Dephosphorylation of a similar protein was commonly observed in cells activated with various stimulants (e.g., phorbol ester, Op.-zym. [16], arachidonic acid [17] and formylpeptide [18,19]). These results suggest that the 21K protein may be a phosphoprotein which is involved in Op.-zym.-provoked activation of the respiratory burst oxidase in intact cells. Recently it was reported that those cytosolic components formed a higher molecular weight complex (molecular weight, 240K–300K) [35]. This 21K protein may function with such complexes.

There are several papers which describe the effect of OA on neutrophil activation [22–26]. Steinbeck et al. observed a synergistic effect of OA (2 and 4 μM for intact cells) and arachidonate [22]. All other researchers used only single doses of OA in a range of micromolar concentrations as an effective dose and it was observed that OA enhanced *N*-formyl-Met-Leu-Phe stimulation [24–26] and inhibited phorbol ester activation [24,25]. Ding and Badwey concentrated on the inhibitory effect of OA on dephosphorylation of $p47^{phox}$ which was phosphorylated by phorbol ester [23]. They concluded that $p47^{phox}$ underwent a continual cycle of phosphorylation and dephosphorylation throughout the period of $O_2^{\cdot-}$ release when phorbol ester was the stimulant. However, we observed that only the phosphorylation of $p47^{phox}$ could not trigger the respiratory burst because OA or calyculin A caused hyperphosphorylation of the $p47^{phox}$ without $O_2^{\cdot-}$ production (unpublished data).

We reported here that 1 μM OA caused an increase in the phosphorylation of $p47^{phox}$ while the Op.-zym.-triggered dephosphorylation of 21K protein still occurred and that 2 μM or higher concentrations of OA inhibited the dephosphorylation of the 21K protein while $p47^{phox}$ and other proteins were heavily phosphorylated. It is possible to speculate that the phosphatase for $p47^{phox}$ was more sensitive to OA than that for the 21K phosphoprotein. In fact, OA is known to be a selective inhibitor of phosphatases, since OA inhibited phosphatase type 2A strongly, type 1 moderately and others weakly [36]. This speculation is also supported by the fact that other phosphatase inhibitors which had no such a selectivity in inhibition of serine phosphatases, calyculin A [36], cyclosporin A, FK506 [37], and vanadate [38] did not have such a biphasic effect as OA.

During the studies on the effect of OA, we found the possibility of involvement of a 21K phosphoprotein in respiratory burst through dephosphorylation. In addition to protein kinases, phosphoprotein phosphatases were recently reported as stimulus-responsive enzymes in other tissues or cells [39–41]. Further studies on the enzymes which catalyze the phosphorylation/dephosphorylation of the 21K protein and a possibility of intracellular transloca-

tion depending on the dephosphorylation are required to clarify its physiological roles.

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